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Role of DC-SIGN in the activation of dendritic cells by HPV-16 L1 virus-like particle vaccine

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Dendritic cell-specific intercellular adhesion molecule-grabbing non-integrin (DC-SIGN), a specific C-type lectin expressed on DC, binds and transmits different pathogens to susceptible cells. In the present study, we examined the role of DC-SIGN in the capture of human papillomavirus (HPV) pseudovirions and activation of DC. We demonstrate that HPV virus-like particles (VLP) bind to DC-SIGN expressed on transfected Raji cells and that antibodies against DC-SIGN block this interaction. DC take up VLP, which activate expression of costimulatory markers and cytokines/chemokines. Although our results indicate that DC-SIGN is not the major receptor for VLP in DC, this interaction contributes to the activation of DC surface antigens (HLA class I) and of various cytokines/chemokines, particularly TNF-α, IL-6, and RANTES. Induction of these markers in DC by VLP was significantly abrogated when binding to DC-SIGN was blocked by anti-DC-SIGN antibodies. These results suggest that DC-SIGN has a functional role in DC activation induced by HPV-16 L1-VLP, and thus highlight new aspects of DC interactions with HPV VLP.

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Introduction

The C-type lectin dendritic cell-specific intercellular adhesion molecule-grabbing non-integrin (DC-SIGN; CD209) is a pathogen recognition receptor that interacts with mannose residues of glycoproteins in a calcium-dependent manner *via* its C-terminal carbohydrate recognition domain [1, 2]. DC-SIGN acts both as an adhesion molecule and pathogen recognition receptor, facilitating DC binding and internalization of several viruses, including HIV-1 [3].

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Abbreviations: DC-SIGN: dendritic cell-specific intercellular adhesion molecule-grabbing non-integrin · **HPV:** human papillomavirus · **IP-10:** IFN-γ-inducible protein 10 · **MCP-1:** monocyte chemotactic protein 1 · **MDDC:** monocyte-derived DC · **MIP-1α:** macrophage inflammatory protein 1α · **VLP:** virus-like particle

In addition to HIV, DC-SIGN was recently shown to bind a variety of microorganisms such as CMV [4], Ebola virus [5], Dengue virus [6], hepatitis C virus [7, 8], simian immunodeficiency virus [9], *Leishmania* [10], *Candida albicans* [11], *Mycobacterium* [12–14] and *Schistosoma* [15]. Some pathogens subvert DC functions to escape immune surveillance [16]. DC-SIGN is abundantly expressed primarily on DC, including those derived from monocytes and those located beneath the genital surface [3].

Human papillomavirus (HPV) virus-like particles (VLP) are a promising vaccine candidate for HPV and cervical cancer [17–20]. Recent clinical trials have shown that VLP afford excellent protection against persistent infection [20, 21]. Because of the lack of a suitable cell culture system for *in vitro* propagation of HPV and the unavailability of virions, HPV VLP have been used as soluble surrogates for native virus particles. The routes of HPV-16 L1-VLP entry in DC and the nature of cellular receptors involved in capture have been studied but remain to be fully characterized. HPV VLP

uptake into human DC has been demonstrated to occur through a clathrin-dependent pathway [22, 23]. VLP bind and activate DC, resulting in up-regulation of costimulatory molecules as well as induction of cytokine release, namely IL-6 and TNF-α [24–26]. Furthermore, interaction with heparan sulfate as an initial step in virus binding to host cells has been described for entry of various viruses, including HPV into various cell types [27].

As human HPV VLP represent a promising vaccine delivery vehicle, delineation of the interaction of VLP with professional antigen-presenting cells may contribute to improved vaccine development. A direct role of DC-SIGN in HPV VLP capture and subsequent activation of DC has not yet been demonstrated. A further characterization of this role may contribute to a better understanding of host/pathogen interactions.

Here, we report that HPV-16 L1-VLP particles bind to DC-SIGN in transfected cell lines, and to a lesser extent in DC, and that this interaction participates in L1-VLP-induced activation of DC. Thus, DC-SIGN is likely involved in DC activation by HPV VLP.

Results and discussion

HPV L1-VLP bind to DC-SIGN in DC-SIGN-transfected cell lines

To study interactions between L1-VLP and DC-SIGN, we first analyzed the binding of VLP to DC-SIGN-transfected or untransfected Raji cell lines using flow cytometry. The transfectants expressed high levels of DC-SIGN (Fig. 1A). We observed a stronger binding of GFP-VLP to DC-SIGN-transfected cells than to untransfected cells (Fig. 1B). Non-specific binding to untransfected cells was also observed, which may be due to binding through other cellular receptors, such as heparan sulfate proteoglycans [27]. The binding of VLP to DC-SIGN-transfected cells was dose dependent (Fig. 1C) and we observed that this binding required calcium (data not shown).

A DC-SIGN-specific blocking monoclonal antibody (10 μg/mL) was used to determine the specificity of this interaction. The binding to DC-SIGN by L1-VLP in transfected cell lines was almost completely blocked in the presence of anti-DC-SIGN antibodies (96 ± 4% inhibition, $n = 3$; Fig. 2A) but not by the IgG2a isotype control antibody. The residual L1-VLP binding observed after pre-incubation with anti-DC-SIGN antibodies was comparable to the unspecific binding level to untransfected cells. These results indicate that the increased binding to the DC-SIGN transfectants was *via* a specific interaction with DC-SIGN. Specificity was further demonstrated in the presence of mannan, which binds to DC-SIGN. As shown in Fig. 2B, pre-incubation of the cells with mannan (120 μg/mL) also inhibited DC-SIGN-mediated binding in the DC-SIGN-transfected Raji cells (91 ± 11% inhibition, $n = 3$), in a similar fashion to what was seen with neutralizing anti-DC-SIGN antibodies. Our results indicate that DC-SIGN might be one of the receptors for L1-VLP uptake.

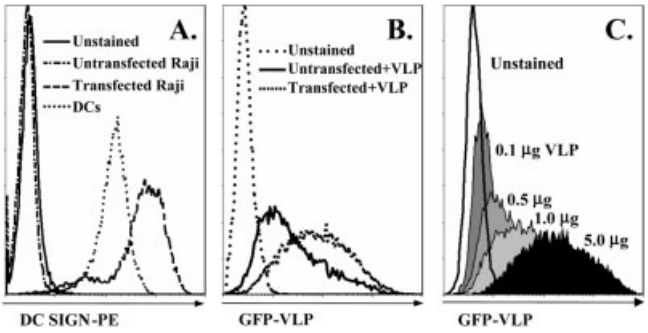

Figure 1. L1-VLP bind to DC-SIGN-transfected Raji cells. (A) DC-SIGN expression on Raji cell lines and immature MDDC. Expression of DC-SIGN was measured by flow cytometry using a DC-SIGN-specific antibody. DC were obtained as described in Materials and methods. (B) L1-VLP bind to DC-SIGN-transfected Raji cells at a stronger intensity than to untransfected cells. Untransfected or DC-SIGN-transfected Raji cells were incubated with GFP-HPV-16 L1-VLP for 1 h at 37°C, as described in Materials and methods. VLP binding is shown as mean fluorescence intensity. (C) VLP binding by DC-SIGN-transfected Raji cells is dose dependent. Concentrations ranging from 0.1 to 5.0 μg GFP-VLP per 1×10^5 cells were incubated for 1 h at 37°C. Cells were washed and analyzed by flow cytometry.

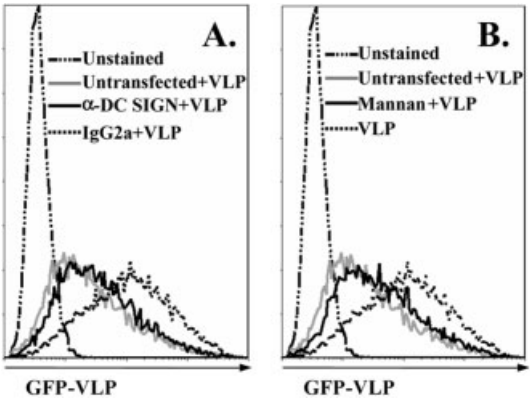

Figure 2. VLP binding to DC-SIGN-transfected Raji cells is blocked by anti-DC-SIGN neutralizing antibodies (A) and mannan (B). Cells were pre-incubated in the presence of anti-DC-SIGN neutralizing antibody or IgG2a isotyope control for 30 min at 37°C before addition of GFP-VLP. The cells were then incubated with the GFP-VLP for 1 h at 37°C. VLP uptake was then determined by FACS and is shown as mean fluorescence intensity. Results with Raji cells are representative of one of three independent experiments.

HPV L1-VLP bind to and activate monocyte-derived DC

Because immature DC express high levels of DC-SIGN, we evaluated the interaction of L1-VLP with immature DC and the potential role of DC-SIGN in this interaction. DC were differentiated from human elutriated monocytes, in the presence of IL-4 and GM-CSF. Immature DC express high levels of DC-SIGN (Fig. 1A). The contribution of DC-SIGN in the binding and entry of L1-VLP into immature DC was investigated using DC-SIGN-specific blocking antibodies. DC showed strong binding to VLP. As seen in Raji cells, this binding was dose dependent (Fig. 3A). Compared to the DC-SIGN-transfected Raji cells, this binding could only be marginally blocked with a specific anti-DC-SIGN antibody ($24 \pm 12\%$ inhibition, $n = 10$ donors; Fig. 3B). Similar levels of blocking were seen when cells were pre-incubated with mannan ($23 \pm 10\%$ inhibition, $n = 8$; Fig. 3C). These results indicate that further mechanisms might be involved in L1-VLP entry into immature DC. These findings are in agreement with previous reports that demonstrate that DC can internalize HPV VLP using other receptors, such as heparan sulfates, CD16 and mannose receptor [23, 24].

Blocking the interaction of L1-VLP with DC-SIGN inhibits VLP-induced activation of DC

It has been previously reported that monocyte-derived DC (MDDC) take up VLP and that VLP are strong adjuvants capable of inducing direct DC activation, leading to an up-regulation of costimulatory molecules and cytokine production [25, 26]. Costimulatory molecules on DC play a critical role in the cascade of events leading to T cell priming. We investigated further the role of DC-SIGN interaction in the VLP-induced expression of costimulatory molecules and cytokines in DC. First, we determined the effect of L1-VLP on DC activation and maturation. Expression of costimulatory cell surface markers and cytokines was determined. Exposure to VLP led to a significant induction of HLA class I antigens ($p = 0.001$) and costimulatory molecules, particularly CD80 and CD86 ($p = 0.001$ and $p = 0.0003$, respectively). Expression of HLA-DR and CD40 was only modestly enhanced and not statistically significant. No significant change was observed for CD83. Flow cytometry histograms for each of the analyzed markers are presented in Fig. 4.

VLP also elicited significant secretion of cytokines (TNF-α, IL-1α, IL-6 and IL-12) (Fig. 5) and chemokines [RANTES, macrophage inflammatory protein 1α (MIP-1α), monocyte chemotactic protein 1 (MCP-1) and IFN-γ-inducible protein 10 (IP-10)] (Fig. 6). Production of IFN-α and IL-8 was induced by L1-VLP, but the levels were not statistically significant ($p = 0.12$ and $p = 0.32$, respectively; see Figs. 5, 6). The induction of all these chemokines in response to HPV-16 L1-VLP has not been previously reported. These results illustrate the complexity of the HPV-16 L1-VLP vaccine effects on DC and they are in agreement with previous findings on the adjuvant effects of L1-VLP on DC [25, 26]. No marked effects of L1-VLP were seen for any of the other cytokines tested that were included in the 22-plex assays (namely IL-1β, IL-2, IL-4, IL-5, IL-7, IL-10, IL-13, IL-15, IL-17, IFN-γ, G-CSF, GM-CSF and Eotaxin; data not shown).

To determine the role of VLP interaction with DC-SIGN in DC activation, DC were treated with anti-DC-SIGN antibodies, or corresponding isotype control antibodies, before incubation with VLP for 24 h. When the interaction of DC-SIGN with L1-VLP was blocked, some inhibition of the expression of several cell surface markers was observed (Fig. 4). However, the inhibition was modest (6–24%) and statistically significant only for MHC class I. These results were consistent with the percentage of blocking of entry in DC shown in Fig. 3.

The blockage of interaction between L1-VLP and DC-SIGN by anti-DC-SIGN antibodies had divergent effects on the induction of several cytokines/chemokines by L1-VLP (Figs. 5, 6). A remarkable inhibition was seen for TNF-α, IL-6 and RANTES (77%, 52% and 39%; $p = 0.0009$, $p = 0.03$ and $p = 0.02$, respectively). A statistically non-significant inhibition was observed for IL-8, IL-12 and MIP-1α, which was in agreement with the levels of blocking of binding by anti-DC-SIGN antibody (42%, 44% and 42%, respectively). A mean inhibition of 89% was seen for IFN-α, but this was not statistically significant ($p = 0.15$) due to the small number of individuals tested ($n = 4$). Although L1-VLP strongly

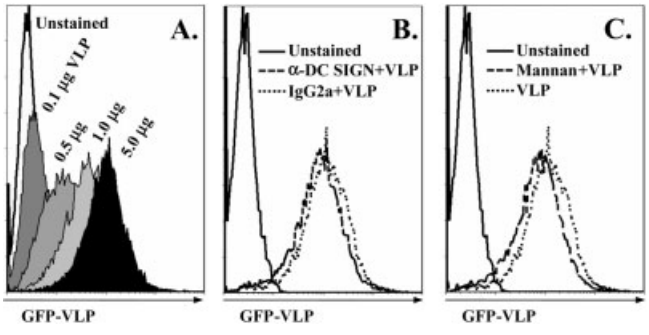

Figure 3. Binding of L1-VLP to immature DC, and binding specificity. (A) GFP-labeled VLP bind strongly to immature DC and this binding is dose dependent. Concentrations ranging from 0.1 to 5 µg GFP-VLP per 8×10^4 cells were incubated for 1 h at 37°C. Cells were washed and analyzed by flow cytometry. (B) Uptake of L1-VLP into DC is only marginally affected by pre-incubation with anti-DC-SIGN-specific antibodies, or with mannan (C). Immature MDDC were obtained as described in Materials and methods. Results shown with DC are representative of one of a total of ten donors tested.

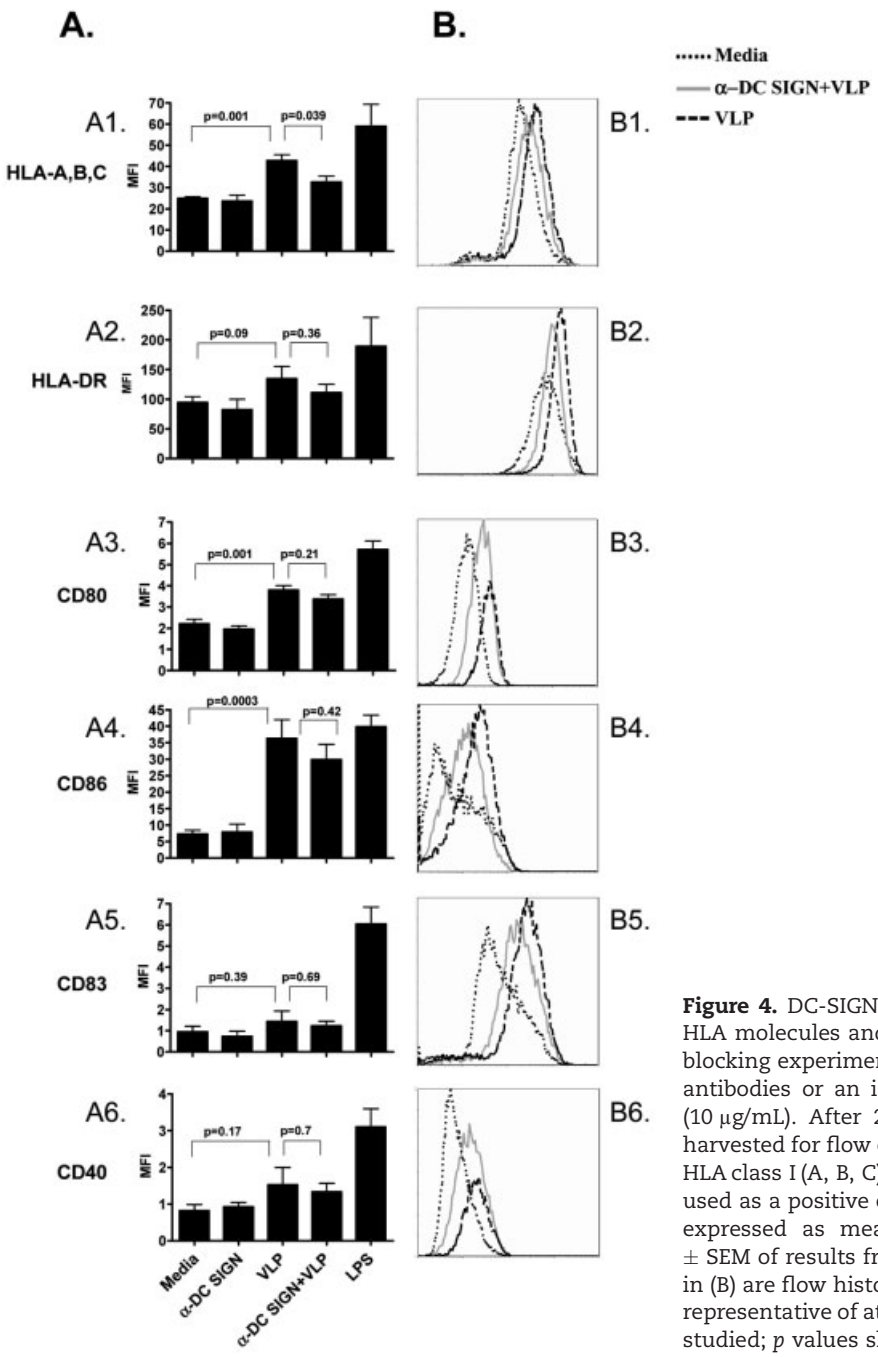

Figure 4. DC-SIGN participates in VLP-induced activation of HLA molecules and costimulatory molecules on DC (A, B). In blocking experiments, DC were pretreated with anti-DC-SIGN antibodies or an isotype control before exposure to L1-VLP (10 μg/mL). After 24 h of incubation with L1-VLP, DC were harvested for flow cytometric analysis of maturation markers: HLA class I (A, B, C), HLA-DR, CD80, CD86, CD83, CD40. LPS was used as a positive control for DC activation. Results in (A) are expressed as mean fluorescence intensity (MFI) averages ± SEM of results from a total of five different donors. Results in (B) are flow histograms obtained by flow cytometry and are representative of at least 5000 cells from one of the five donors studied; *p* values shown were determined using a *t*-test.

induced secretion of IL-1α, MCP-1 and IP-10, no inhibition in their production was observed when the interaction of VLP with DC-SIGN was blocked with anti-DC-SIGN antibodies. These results indicate that the induction of these cytokines/chemokines by L1-VLP is not DC-SIGN dependent.

Effects of blocking the interaction of L1-VLP with DC-SIGN on allogeneic and autologous L1-VLP responses *in vitro*

Next, the effect of L1-VLP-pulsed DC on T cell proliferation in an allogeneic mixed lymphocyte reaction was investigated, as a measure of DC function. As shown in Fig. 7A, DC stimulated with L1-VLP promoted an increase in allogeneic T cell proliferation (average 1.9-fold increase over response in the absence of VLP). In addition, a strong T cell response to L1-VLP was observed when T cells were co-cultured with L1-VLP-pulsed autologous DC (average of 15-fold increase over response to DC pulsed with medium, $p = 0.008$, $n = 6$). These results are in agreement with previous studies [28].

Since DC-SIGN appeared to be involved in activation of MHC class I antigen and cytokine responses in DC, we examined the effect of DC pulsed with L1-VLP in the

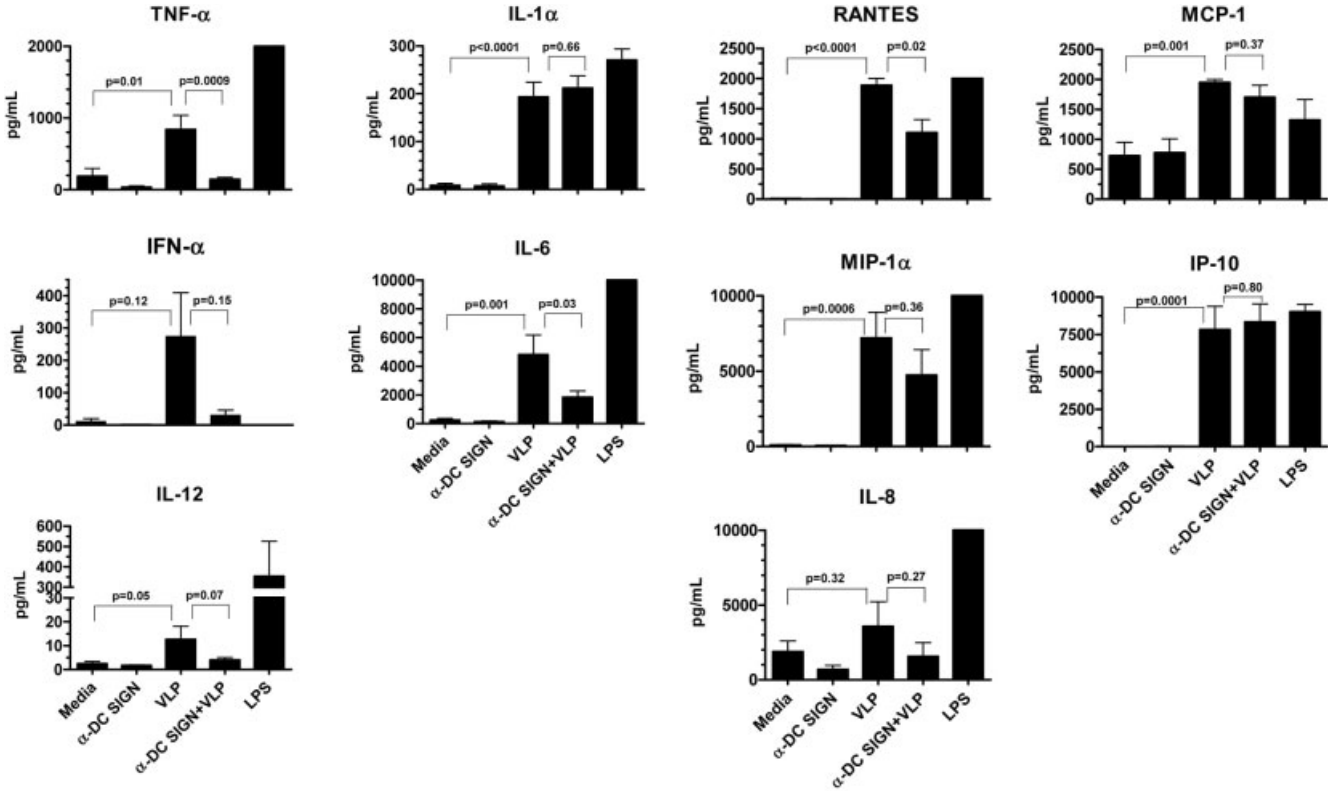

Figure 5. L1-VLP induce activation of several cytokines in immature MDDC and the L1-VLP interaction with DC-SIGN is necessary for their activation. DC were pretreated with anti-DC-SIGN antibodies, isotype control antibodies or medium alone, before exposure to L1-VLP (10 μg/mL). After 24 h of incubation with L1-VLP, supernatants of these cultures were collected for cytokine quantitation using multiplex cytokine kits. Data are presented as averages of cytokine production in pg/mL ± SEM of experiments performed in a total of five healthy blood bank donors. LPS-treated DC were used as positive control; *p* values shown were determined using a *t*-test.

Figure 6. L1-VLP induce activation of chemokines in immature MDDC and the L1-VLP interaction with DC-SIGN is necessary for their activation. DC were pretreated with anti-DC-SIGN antibodies, isotype control antibodies or medium alone, before exposure to L1-VLP. After 24 h of incubation with L1-VLP, supernatants of these cultures were collected for chemokine quantitation using multiplex kits. Data are presented as averages of chemokine production in pg/mL ± SEM of experiments performed in a total of five healthy blood bank donors. LPS-treated DC were used as positive control; *p* values shown were determined using a *t*-test.

presence of an anti-DC-SIGN monoclonal antibody on allogeneic T cell responses. When the interaction of L1-VLP with DC-SIGN was blocked, there was a reduction of approximately 40% in T cell allostimulatory activity induced by L1-VLP-pulsed DC. These results could be due to the decreased MHC class I expression observed in DC pulsed with VLP in the presence of anti-DC-SIGN antibodies. Blocking of DC-SIGN had a different effect on autologous T cell proliferative responses to L1-VLP. The proliferative response of purified T cells after incubation with DC pulsed with L1-VLP in the presence of anti-DC-SIGN antibodies was higher, although not statistically significant, than with DC incubated with VLP alone (approximately 35% increase). These results raise the possibility that DC-SIGN can differentially affect T cell proliferation in these two different types of *in vitro* stimulation, although these aspects cannot be addressed in the present study. Interestingly, a recent study indicates that DC-SIGN can differentially modulate T cell stimulation under suboptimal *versus* optimal T cell signaling [29].

Cytokine profiles in co-cultures of purified T cells and DC were determined using a multiplex system for 23 different cytokines (Fig. 7B). In autologous co-cultures, we observed an increase in T cell-derived cytokines (IL-2, IFN-γ and IL-13), as well as cytokines/chemokines induced in DC-purified cultures (TNF-α, MIP-1α, RANTES and IL-6; $p > 0.05$ for all, except RANTES with $p = 0.009$).

As observed in DC-purified cultures (Figs. 5, 6), blocking DC-SIGN interaction inhibited cytokines typically produced by L1-VLP-stimulated DC ($p = 0.03$ for TNF-α and $p = 0.04$ for RANTES) (Fig. 7). Similar trends for these cytokines were observed in allogeneic T cell cultures (data not shown).

However, although these autologous co-cultures contained lower levels of inflammatory cytokines/chemokines, no inhibition of T cell cytokine production

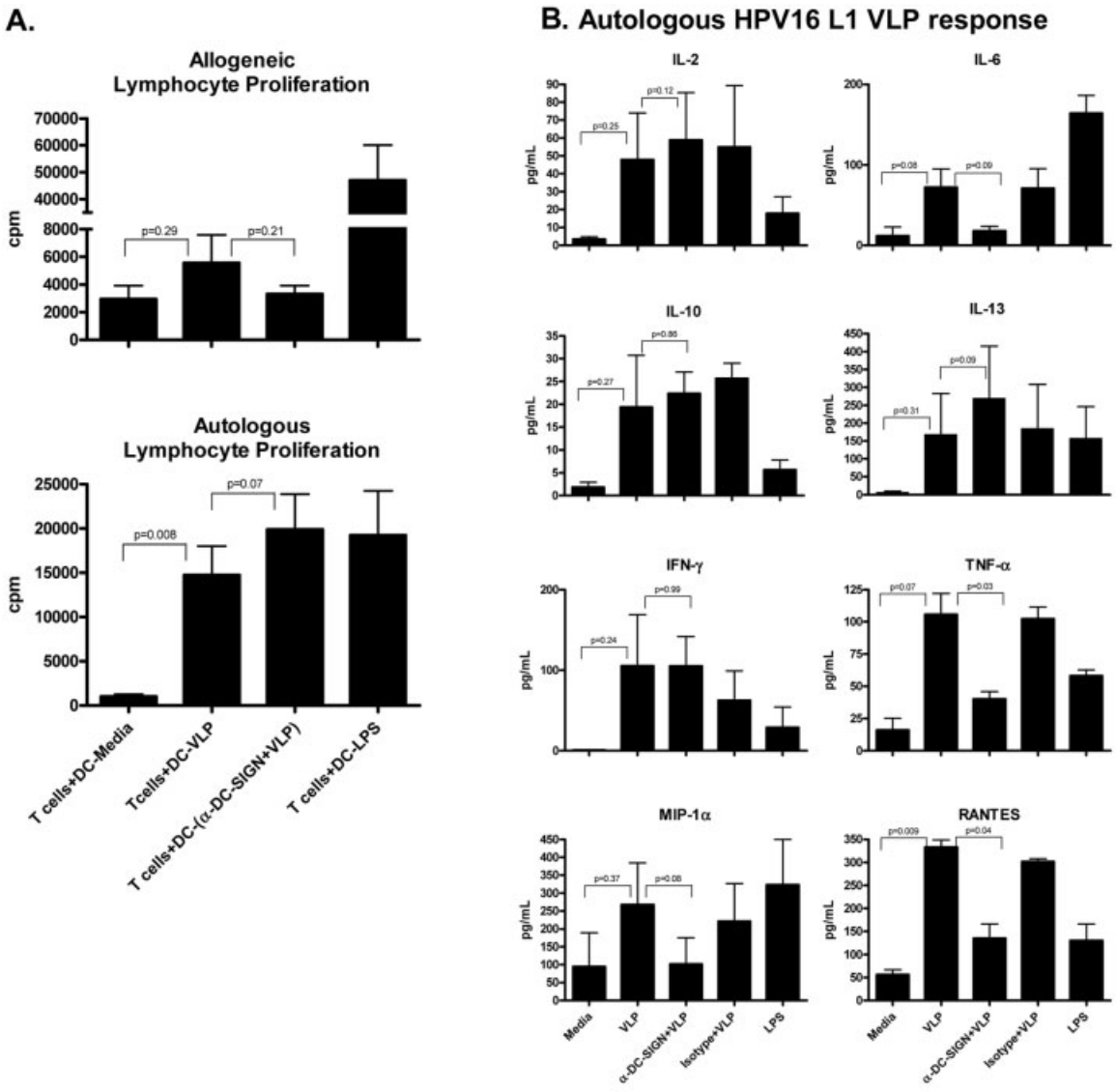

Figure 7. Effect of DC-SIGN blocking on allogeneic and autologous T cell responses induced by HPV-16 L1-VLP-pulsed DC. DC (2×10^5) were stimulated with medium, LPS (controls), and HPV-16 L1-VLP with or without pre-incubation with anti-DC-SIGN antibody or an isotype control, as indicated. After 24 h, DC were co-cultured at a 1 : 50 ratio with purified allogeneic T cells or at a 1 : 20 ratio with purified autologous T cells, and incubated for 5 days. T cell proliferation was assayed by [^{3}H]thymidine incorporation (A). Supernatants from the autologous cultures were collected before pulsing, and cytokine content was determined using multiplex cytokine analysis (B). The results are expressed as means ± SD of six (A) or three (B) independent experiments; *p* values shown were determined using a paired *t*-test.

was observed (IL-2, IFN-γ, IL-10, IL-13). In fact, slight non-significant increases in these T cell cytokines were observed. In the 5-day allogeneic cultures, the levels of most of these cytokines were too low to draw conclusions (data not shown).

Future studies are needed to better address and understand the role of DC-SIGN in T cell responses to L1-VLP. Since utilization of blocking antibodies presents its own limitations, studies are underway in our laboratory using siRNA for DC-SIGN, to better characterize its role in DC activation by L1-VLP.

The interesting observation that the blockage of L1-VLP binding to DC-SIGN has a differential effect on cytokine and chemokine expression suggests that there is more than one pathway of interaction of L1-VLP with DC and that the observed response may be a consequence of the combination of all these interactions. The delineation of the impact of these interactions in immune responses is therefore warranted.

Our findings indicate for the first time that DC-SIGN plays a role in DC activation/maturation by L1-VLP. Interaction of L1-VLP with DC-SIGN on DC may initiate

signaling events, resulting in activation of costimulatory molecules and a variety of inflammatory and/or antiviral cytokines. Signal transduction upon ligand binding to DC-SIGN has not yet been proven, although the presence of an intracellular immunoreceptor tyrosine-based activation motif (ITAM) similar to dectin-1 suggests that DC-SIGN could be a signaling receptor [30]. Additionally, interactions of DC-SIGN with certain pathogens may have direct consequences for Th1/Th2 polarization [31]. Interestingly, blocking of the interaction between DC and T cells with an anti-DC-SIGN antibody influences allostimulatory properties in T cells [1]. Several recent studies have demonstrated that cross-talk between C-type lectins and TLR can occur [14, 32, 33], suggesting that simultaneous interaction of pathogens with both receptors can modulate the response by DC. In this context, a recent study indicates that activation of DC by HPV L1-VLP is MyD88 dependent [34]. Polymorphisms in the DC-SIGN promoter were recently reported [35], but their effect on DC-SIGN binding and signaling has not yet been examined.

Concluding remarks

In conclusion, this study demonstrates that HPV VLP bind to DC-SIGN and that this interaction participates in the activation of secretion of some cytokines and chemokines by DC in response to L1-VLP, without affecting others. In addition, this interaction does not appear to be essential for DC activation of T cell proliferative responses to L1-VLP *in vitro*. Thus, this study adds HPV VLP to the growing list of antigens that functionally interact with DC-SIGN and suggests the involvement of multiple mechanisms of L1-VLP recognition and signaling in DC. The potential *in vivo* impact of these *in vitro* observations remains to be demonstrated. Further delineation of the receptors and pathways involved in the interaction of VLP with professional antigen-presenting cells may contribute to a better understanding of vaccine immunogenicity and host/pathogen interactions.

Materials and methods

Cells

Stable Raji transfectants expressing wild-type DC-SIGN were generated as described [36]. Immature DC were obtained as described before [37]. In short, elutriated monocytes obtained from the NIH blood bank (Bethesda, MD) were subjected to an adherence step for 2 h. Adherent monocytes were differentiated into immature DC in the presence of IL-4 (50 ng/mL; Peprotech, Rocky Hill, NJ) and GM-CSF (Leukine, 100 U/mL; Berlex, Inc., Montville, NJ). Medium was changed every 3 days. At day 6–7, the phenotype of the cultured DC was confirmed by flow cytometric analysis (at least 5000 cells were counted) [38]. The DC expressed high levels of DC-SIGN, CD11c, CD1a and were negative for CD14, CD3 and lineage markers (data not shown). DC-SIGN, CD11c, CD1a and lineage markers were provided by Becton Dickinson (San Jose, CA), CD14 and CD3 by Beckman Coulter (Fullerton, CA).

VLP binding/entry assays

GFP-VLP were provided by Dr. J. T. Schiller (NIH, Bethesda, MD) and consisted of HPV-16 L1-VLP containing L2 bovine papillomavirus-GFP fusion proteins. These were produced as reported [26]. Stable Raji transfectants expressing wild-type DC-SIGN as well as parental Raji cell controls were incubated with GFP-VLP (10 μg/mL) or medium for 1 h at 37°C in PBS supplemented with 2% FCS (Invitrogen, Life Technologies) and 1 mM $CaCl_2$ (Sigma) (assay buffer). After incubation, cells were washed, resuspended in assay buffer and analyzed by flow cytometry (at least 5000 cells were counted). In similar experiments, immature DC were incubated with GFP-L1-VLP for 1 h at 37°C in the assay buffer. Specificity was evaluated using a neutralizing anti-DC-SIGN antibody (R&D Systems, Minneapolis, MN) or mannan (Sigma-Aldrich, St. Louis, MO), known to block the binding of DC-SIGN ligands. In neutralization assays, the antibodies or mannan were added 30 min before addition of GFP-VLP and incubated at 37°C. Microscope fluorescence observation of stained DC and Raji cells demonstrated that GFP-VLP was internalized by these cells (data not shown).

DC activation

Immature DC were incubated in the presence or absence of HPV-16 VLP (10 μg/mL), or lipopolysaccharide (LPS, 2 μg/mL; Sigma-Aldrich, St. Louis, MO) as a positive control for 24 h. The recombinant L1-VLP were expressed in a baculovirus system (Novavax, Rockville, MD) and produced for clinical usage as reported [39]. In neutralization assays, the antibodies were added 30 min before the addition of L1-VLP and incubated at 37°C. After incubation, cell-free supernatants were collected for cytokine analysis and cells were washed and analyzed by flow cytometry (FC500 Flow Cytometer; Beckman Coulter) for the expression of several surface markers (CD1a, CD3, CD14, CD11c, HLA class I, HLA class II, CD80, CD83, CD86, CD40 and appropriate isotype controls; all from Becton Dickinson, except CD3 and CD14 that were from Beckman Coulter). DC viability was assessed by staining with 7-amino-actinomycin D (7-AAD; Beckman Coulter), and it was greater than 96% in all cases. Supernatants were tested using commercially available kits for multiplex cytokine analysis (22-plex; Linco Research, Inc., St. Louis, MO), containing: IL-1β, IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-10, IL-12p70, IL-13, IL-15, IL-17, IL-1α, IFN-γ, G-CSF, GM-CSF, TNF-α, Eotaxin, MCP-1, MIP-1α, IP-10 and RANTES. IFN-α was analyzed separately using a commercially available kit (Biosource International, Camarillo, CA). Only the cytokines induced by L1-VLP stimulation are shown. Results were analyzed in the Bioplex instrument (Bio-Rad, Hercules, CA). Results are expressed in

pg/mL and correspond to the average of duplicate measurements. The lowest level of detection was 3.2 pg/mL for all cytokines tested. Levels lower than the lowest detection levels were arbitrarily considered to be one half of the lowest detection level (1.6 pg/mL).

Allogeneic and autologous L1-VLP T cell proliferation

Human T cells were enriched from cryopreserved PBMC by negative selection using the Dynal T cell negative isolation kit (Dynal Biotech, Oslo, Norway) according to the manufacturer's recommendations. Purity was higher than 91% in all cases. Allogeneic DC stimulator cells were pulsed for 24 h with (a) medium (control), (b) L1-VLP alone, (c) L1-VLP that were pre-incubated with anti-DC-SIGN antibodies or an isotype control antibody, and (d) LPS as a positive control. DC were subsequently washed twice, resuspended in AIM V medium (Invitrogen, New York, NY) and added to purified T cells from allogeneic donors in 96-well round-bottom microtiter plates in a total volume of 200 μL (ratio DC/T cells 1 : 50). In the case of autologous T cell proliferative response to L1-VLP, DC treated as above were added to purified autologous T cells at a ratio of 1 : 20 in a total volume of 200 μL. Triplicate cultures were incubated at 37°C, 5% CO_2 for 5 days. Supernatants were then collected and stored frozen until cytokine analysis, and cultures were pulsed with [^{3}H]thymidine (1 μCi/well), harvested 18 h later, and incorporation of [^{3}H]thymidine was measured in a beta counter (Wallac, Boston, MA). Results are represented as cpm ± SD of triplicate wells.

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References

1 **Geijtenbeek, T. B., Torensma, R., van Vliet, S. J., van Duijnhoven, G. C., Adema, G. J., van Kooyk, Y. and Figdor, C. G.,** Identification of DC-SIGN, a novel dendritic cell-specific ICAM-3 receptor that supports primary immune responses. *Cell* 2000. **100:** 575–585.

2 **Steinman, R. M.,** DC-SIGN: a guide to some mysteries of dendritic cells. *Cell* 2000. **100:** 491–494.

3 **Geijtenbeek, T. B., Kwon, D. S., Torensma, R., van Vliet, S. J., van Duijnhoven, G. C., Middel, J., Cornelissen, I. L. *et al.*,** DC-SIGN, a dendritic cell-specific HIV-1-binding protein that enhances *trans*-infection of T cells. *Cell* 2000. **100:** 587–597.

4 **Halary, F., Amara, A., Lortat-Jacob, H., Messerle, M., Delaunay, T., Houles, C., Fieschi, F. *et al.*,** Human cytomegalovirus binding to DC-SIGN is required for dendritic cell infection and target cell *trans*-infection. *Immunity* 2002. **17:** 653–664.

5 **Alvarez, C. P., Lasala, F., Carrillo, J., Muniz, O., Corbi, A. L. and Delgado, R.,** C-type lectins DC-SIGN and L-SIGN mediate cellular entry by Ebola virus in *cis* and in *trans*. *J. Virol.* 2002. **76:** 6841–6844.

6 **Tassaneetrithep, B., Burgess, T. H., Granelli-Piperno, A., Trumpfheller, C., Finke, J., Sun, W., Eller, M. A. *et al.*,** DC-SIGN (CD209) mediates Dengue virus infection of human dendritic cells. *J. Exp. Med.* 2003. **197:** 823–829.

7 **Lozach, P. Y., Lortat-Jacob, H., de Lacroix de Lavalette, A., Staropoli, I., Foung, S., Amara, A., Houles, C. *et al.*,** DC-SIGN and L-SIGN are high affinity binding receptors for hepatitis C virus glycoprotein E2. *J. Biol. Chem.* 2003. **278:** 20358–20366.

8 **Pohlmann, S., Zhang, J., Baribaud, F., Chen, Z., Leslie, G. J., Lin, G., Granelli-Piperno, A. *et al.*,** Hepatitis C virus glycoproteins interact with DC-SIGN and DC-SIGNR. *J. Virol.* 2003. **77:** 4070–4080.

9 **Pohlmann, S., Baribaud, F., Lee, B., Leslie, G. J., Sanchez, M. D., Hiebenthal-Millow, K., Munch, J. *et al.*,** DC-SIGN interactions with human immunodeficiency virus type 1 and 2 and simian immunodeficiency virus. *J. Virol.* 2001. **75:** 4664–4672.

10 **Colmenares, M., Puig-Kroger, A., Pello, O. M., Corbi, A. L. and Rivas, L.,** Dendritic cell (DC)-specific intercellular adhesion molecule 3 (ICAM-3)-grabbing nonintegrin (DC-SIGN, CD209), a C-type surface lectin in human DCs, is a receptor for *Leishmania* amastigotes. *J. Biol. Chem.* 2002. **277:** 36766–36769.

11 **Cambi, A., Gijzen, K., de Vries, J. M., Torensma, R., Joosten, B., Adema, G. J., Netea, M. G. *et al.*,** The C-type lectin DC-SIGN (CD209) is an antigen-uptake receptor for *Candida albicans* on dendritic cells. *Eur. J. Immunol.* 2003. **33:** 532–538.

12 **Tailleux, L., Schwartz, O., Herrmann, J. L., Pivert, E., Jackson, M., Amara, A., Legres, L. *et al.*,** DC-SIGN is the major *Mycobacterium tuberculosis* receptor on human dendritic cells. *J. Exp. Med.* 2003. **197:** 121–127.

13 **Maeda, N., Nigou, J., Herrmann, J. L., Jackson, M., Amara, A., Lagrange, P. H., Puzo, G. *et al.*,** The cell surface receptor DC-SIGN discriminates between *Mycobacterium* species through selective recognition of the mannose caps on lipoarabinomannan. *J. Biol. Chem.* 2003. **278:** 5513–5516.

14 **Geijtenbeek, T. B., Van Vliet, S. J., Koppel, E. A., Sanchez-Hernandez, M., Vandenbroucke-Grauls, C. M., Appelmelk, B. and Van Kooyk, Y.,** Mycobacteria target DC-SIGN to suppress dendritic cell function. *J. Exp. Med.* 2003. **197:** 7–17.

15 **van Die, I., van Vliet, S. J., Nyame, A. K., Cummings, R. D., Bank, C. M., Appelmelk, B., Geijtenbeek, T. B. *et al.*,** The dendritic cell-specific C-type lectin DC-SIGN is a receptor for *Schistosoma mansoni* egg antigens and recognizes the glycan antigen Lewis X. *Glycobiology* 2003. **13:** 471–478.

16 **Ludwig, I. S., Lekkerkerker, A. N., Depla, E., Bosman, F., Musters, R. J., Depraetere, S., van Kooyk, Y. *et al.*,** Hepatitis C virus targets DC-SIGN and L-SIGN to escape lysosomal degradation. *J. Virol.* 2004. **78:** 8322–8332.

17 **Schiller, J. T. and Lowy, D. R.,** Papillomavirus-like particle based vaccines: Cervical cancer and beyond. *Expert Opin. Biol. Ther.* 2001. **1:** 571–581.

18 **Stanley, M. A.,** Human papillomavirus vaccines. *Curr. Opin. Mol. Ther.* 2002. **4:** 15–22.

19 **Brown, D. R., Fife, K. H., Wheeler, C. M., Koutsky, L. A., Lupinacci, L. M., Railkar, R., Suhr, G. *et al.*,** Early assessment of the efficacy of a human papillomavirus type 16 L1 virus-like particle vaccine. *Vaccine* 2004. **22:** 2936–2942.

20 **Koutsky, L. A., Ault, K. A., Wheeler, C. M., Brown, D. R., Barr, E., Alvarez, F. B., Chiacchierini, L. M. *et al.*,** A controlled trial of a human papillomavirus type 16 vaccine. *N. Engl. J. Med.* 2002. **347:** 1645–1651.

21 **Harper, D. M., Franco, E. L., Wheeler, C., Ferris, D. G., Jenkins, D., Schuind, A., Zahaf, T. *et al.*,** Efficacy of a bivalent L1 virus-like particle vaccine in prevention of infection with human papillomavirus types 16 and 18 in young women: A randomised controlled trial. *Lancet* 2004. **364:** 1757–1765.

22 **Day, P. M., Lowy, D. R. and Schiller, J. T.,** Papillomaviruses infect cells *via* a clathrin-dependent pathway. *Virology* 2003. **307:** 1–11.

23 **Yan, M., Peng, J., Jabbar, I. A., Liu, X., Filgueira, L., Frazer, I. H. and Thomas, R.,** Despite differences between dendritic cells and Langerhans cells in the mechanism of papillomavirus-like particle antigen uptake, both cells cross-prime T cells. *Virology* 2004. **324:** 297–310.

24 **Da Silva, D. M., Velders, M. P., Nieland, J. D., Schiller, J. T., Nickoloff, B. J. and Kast, W. M.,** Physical interaction of human papillomavirus virus-like particles with immune cells. *Int. Immunol.* 2001. **13:** 633–641.

25 **Fausch, S. C., Da Silva, D. M. and Kast, W. M.,** Differential uptake and cross-presentation of human papillomavirus virus-like particles by dendritic cells and Langerhans cells. *Cancer Res.* 2003. **63:** 3478–3482.

26 **Lenz, P., Day, P. M., Pang, Y. Y., Frye, S. A., Jensen, P. N., Lowy, D. R. and Schiller, J. T.,** Papillomavirus-like particles induce acute activation of dendritic cells. *J. Immunol.* 2001. **166:** 5346–5355.

27 **Joyce, J. G., Tung, J. S., Przysiecki, C. T., Cook, J. C., Lehman, E. D., Sands, J. A., Jansen, K. U. *et al.*,** The L1 major capsid protein of human papillomavirus type 11 recombinant virus-like particles interacts with heparin and cell-surface glycosaminoglycans on human keratinocytes. *J. Biol. Chem.* 1999. **274:** 5810–5822.

28 **Fausch, S. C., Da Silva, D. M., Rudolf, M. P. and Kast, W. M.,** Human papillomavirus virus-like particles do not activate Langerhans cells: A possible immune escape mechanism used by human papillomaviruses. *J. Immunol.* 2002. **169:** 3242–3249.

29 **Martinez, O., Brackenridge, S., El-Idrissi Mel, A. and Prabhakar, B. S.,** DC-SIGN, but not sDC-SIGN, can modulate IL-2 production from PMA- and anti-CD3-stimulated primary human CD4 T cells. *Int. Immunol.* 2005. **17:** 769–778.

30 **Gantner, B. N., Simmons, R. M., Canavera, S. J., Akira, S. and Underhill, D. M.,** Collaborative induction of inflammatory responses by dectin-1 and Toll-like receptor 2. *J. Exp. Med.* 2003. **197:** 1107–1117.

31 **Bergman, M. P., Engering, A., Smits, H. H., van Vliet, S. J., van Bodegraven, A. A., Wirth, H. P., Kapsenberg, M. L. *et al.*,** *Helicobacter pylori* modulates the T helper cell 1/T helper cell 2 balance through phase-variable interaction between lipopolysaccharide and DC-SIGN. *J. Exp. Med.* 2004. **200:** 979–990.

32 **Gardner, J. P., Durso, R. J., Arrigale, R. R., Donovan, G. P., Maddon, P. J., Dragic, T. and Olson, W. C.,** L-SIGN (CD 209L) is a liver-specific capture receptor for hepatitis C virus. *Proc. Natl. Acad. Sci. USA* 2003. **100:** 4498–4503.

33 **Nigou, J., Zelle-Rieser, C., Gilleron, M., Thurnher, M. and Puzo, G.,** Mannosylated lipoarabinomannans inhibit IL-12 production by human dendritic cells: Evidence for a negative signal delivered through the mannose receptor. *J. Immunol.* 2001. **166:** 7477–7485.

34 **Yang, R., Murillo, F. M., Cui, H., Blosser, R., Uematsu, S., Takeda, K., Akira, S. *et al.*,** Papillomavirus-like particles stimulate murine bone marrow-derived dendritic cells to produce alpha interferon and Th1 immune responses *via* MyD88. *J. Virol.* 2004. **78:** 11152–11160.

35 **Martin, M. P., Lederman, M. M., Hutcheson, H. B., Goedert, J. J., Nelson, G. W., van Kooyk, Y., Detels, R. *et al.*,** Association of DC-SIGN promoter polymorphism with increased risk for parenteral, but not mucosal, acquisition of human immunodeficiency virus type 1 infection. *J. Virol.* 2004. **78:** 14053–14056.

36 **Wu, L., Martin, T. D., Carrington, M. and KewalRamani, V. N.,** Raji B cells, misidentified as THP-1 cells, stimulate DC-SIGN-mediated HIV transmission. *Virology* 2004. **318:** 17–23.

37 **Nelson, E. L., Strobl, S., Subleski, J., Prieto, D., Kopp, W. C. and Nelson, P. J.,** Cycling of human dendritic cell effector phenotypes in response to TNF-alpha: Modification of the current 'maturation' paradigm and implications for *in vivo* immunoregulation. *FASEB J.* 1999. **13:** 2021–2030.

38 **Pinto, L. A., Trivett, M. T., Wallace, D., Higgins, J., Baseler, M., Terabe, M., Belyakov, I. M. *et al.*,** Fixation and cryopreservation of whole blood and isolated mononuclear cells: Influence of different procedures on lymphocyte subset analysis by flow cytometry. *Cytometry B Clin. Cytom.* 2005. **63B:** 47–55.

39 **Pinto, L. A., Edwards, J., Castle, P. E., Harro, C. D., Lowy, D. R., Schiller, J. T., Wallace, D. *et al.*,** Cellular immune responses to human papillomavirus (HPV)-16 L1 in healthy volunteers immunized with recombinant HPV-16 L1 virus-like particles. *J. Infect. Dis.* 2003. **188:** 327–338.

